REMARKS

Applicants wish to thank the Examiner for the attention accorded to the instant application, and respectfully requests reconsideration of the application as amended. Claims 1-18 are pending in the present application. Claims 1 and 18 have been amended to recite a step of preparing a mixture containing a series of reaction products that are obtained from an original peptide to be examined by releasing the C-terminal amino acids successively by chemical means and the remains of the original peptide. Claims 1, 6, 7 and 18 have been amended to recite that the formation of the 5-oxazolone structure and the cleavage of the 5-oxazolone ring are carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of the catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids. Claims 1, 6, 7 and 18 have been amended to recite that the hydrolysis is carried out by using water molecules in the presence of a catalytic amount of the basic, nitrogen-containing, aromatic compound or the tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS. Claim 7 has been amended to recite a polyacrylamide gel carrier.

Support for these amendments can be found throughout the application, such as in page 18 line 3-page 19 line 17, page 113 lines 3-9 and page 128 line 14-page 129 line 17. No new matter has been entered into the disclosure by way of the above amendments.

Claims 1, 2, 4-6 and 18 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Tsugita, Akira et. al., "Additional Possible tools for identification of proteins on one or two dimensional electrophoresis", 1998, Electrophoresis, Vol. 19, pages 928-938 (hereinafter

"Tsugita") in view of Covey et. al. U.S Patent No. 5,952,653 (hereinafter "Covey") and Xu, Naxing et. al., "Structural characterization of peptidoglycan muropeptides by matrix-assisted laser desorption ionization mass spectrometry and postsource decay analysis," 1997, Analytical Biochemistry, Vol. 248, page 7-14 (hereinafter "Xu").

Claim 3 stands rejected under 35 U.S.C. 103(a) as being unpatentable over Tsugita in view of Covey and Xu, as applied to Claims 1-2, 4-6 and 18 above, and further in view of Harris, William A., et. al., "Use of matrix clusters and trypsin autolysis fragments as mass calibrants in matrix assisted laser desorption/ionization time-of-flight mass spectrometry," 2002, Rapid Communications in Mass Spectrometry, vol. 16, pages 1714-1722 (hereinafter "Harris").

Claims 7-17 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Tsugita in view of Covey and Xu as applied to 1, 2, 4-6 and 18 above, and further in view of Vogt, S. et. al., "Effective esterification of carboxymethyl cellulose in a new non-aqueous swelling system," 1996, Polymer Bulletin, Vol. 36, page 549-555 (hereinafter "Vogt").

In view of the following remarks, Applicants request further examination and reconsideration of the present patent application.

Rejections under 35 U.S.C. §103

Claims 1, 2, 4-6 and 18 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Tsugita in view of Covey and Xu. This rejection should be withdrawn based on the comments and remarks herein.

The combination of Tsugita, Covey and Xu do not render the claimed invention obvious for at least the following reasons. None of the references teach or suggest the formation of a 5-oxazolone structure where the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the

presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, none of the references teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Tsugita teaches and suggests a process for C-terminal sequencing for the protein, as disclosed in the Section "2.13 C-terminal sequencing", in which the C-terminal amino acid liberated from the denatured protein (peptide) is modified with fluorescein isothiocynated and analyzed by HPLC, thereby the C-terminal sequence of the denatured protein (peptide) is analyzed without use of mass-spectroscopic analysis. (See "2.13 C-terminal sequencing" of Tsugita)

The process of "2.13 C-terminal sequencing", as taught and suggested by Tsugita, comprises the following steps:

The first step of extracting the denatured protein (peptide) from the protein spot on the polyacrylamide gel is carried out by using the following extraction procedure.

The protein spot is excised from the polyacrylamide gel and broken up by the use of a small hand-held homogenizer after addition to the 500 μL of 6M guanidine-HCl, 0.1% SDS, 0.5 M Bicine (2-(Bis(2-hydroxyethyl)amino)acetic acid), 4mM EDTA, pH 8.0-8.5. The 6M

guanidine-HCl and 0.1% SDS contained in the solution is successfully used to denature the protein, and thus the denatured protein can be easily extracted from the gel carrier to be collect in the pool of supernatant and two addition washes.

The denatured protein contained in the pooled supernatant is subjected to purification and separation with the use of a mini-column of C18 silica. The denatured protein isolated by the column separation method is dried up to use as the dried protein sample.

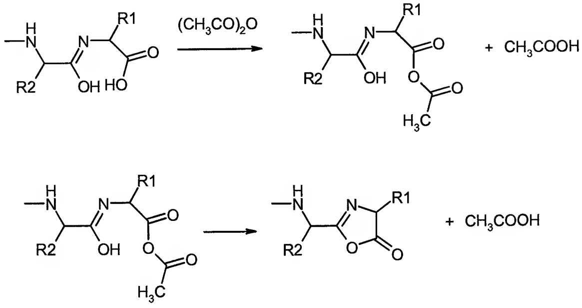
Therefore, Tsugita fails to teach or suggest any process for C-terminal sequencing in which the reactions for C-terminal stepwise degradation are carried out for the peptide being maintained in a state that it is bound on the gel carrier, in particular, on a polyacrylamide gel, as recited in Claims 1, 18 and all claims depending therefrom of the present application. In contrast, Tsugita teaches the need to extract the protein from a gel carrier prior to degradation.

In "2.13 C-terminal sequencing", Tsugita teaches and suggests a series of reactions for C-terminal stepwise degradation used for the dried protein sample comprising the following three reaction sub-steps (i) – (iii):

(i) The first reaction sub-step includes acetylation of the N-terminus of the peptide (denatured protein) and formation of an oxazolone at the C-terminal carboxyl group of the peptide (denatured protein):

Acetic anhydride with 20% acetic acid tetrahydrofuran solution in the presence of 1% DTT was reacted in liquid phase on the dried sample of peptide (denatured protein) at 60 °C for 10 min. The reaction which forms the oxazolone may be carried out by the following reaction scheme:

Formation of the oxazolone at the C-terminal carboxyl group:



The acetic acid may be used as a catalyst for inducing the convention of keto-form of the amido moiety into the enol-form.

However, Tsugita fails to provide any evidence suggesting that N-acetylation on the amino group of Lys residue and O-acetylation on the hydroxyl group of Ser and Thr residues as well as N-acetylation on the N-terminus are achieved at the first reaction sub-step.

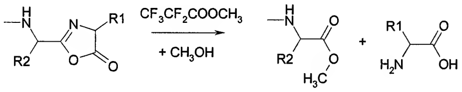
Further, at the least, Tsugita fails to provide any evidence suggesting that acetic anhydride with 20% acetic acid tetrahydrofuran solution would be successfully used in the absence of DTT for N-acetylation on the amino group of Lys residue as well as N-acetylation on the N-terminus.

(ii) The second reaction sub-step for degradation of the oxazolone-ring to liberate the C-terminal amino acid and to form the esterified peptide:

The liquid phase reaction is made with 5% PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3) in methanol (CH_3OH) at 5 °C for 15 min.

The reaction of degradation of the oxazolone ring may be carried out by the following reaction scheme:

<u>Degradation of the oxazolone:</u>



The reaction mechanism may be alcoholysis in help of catalytic function of PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3). The C-terminal amino acid was liberated to be dissolved in the methanol solution, and thus, the peptidyl reaction product was formed in the shape of esterified peptide.

Therefore, PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3) was used as a catalytic agent for inducing the solvolysis reaction with use of methanol (CH_3OH) on the oxazolone-ring.

In view of this, Tsugita fails to teach or suggest any reaction for degradation of the oxazolone-ring which would be achieved without use of methanol (CH_3OH).

Furthermore, Tsugita fails to provide any teaching or suggestion that PEPA (pentafluoropropionic acid: CF_3CF_2-COOH) without methanol (CH_3OH) would be used as

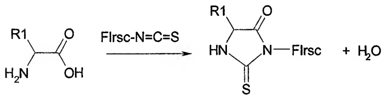
a reactant for the degradation of the oxazolone, in place of PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3) with methanol (CH_3OH). Methanol (CH_3OH) is a well-known protic solvent that is a suitable reactant for alcoholysis reaction.

Therefore, Tsugita fails to provide any teaching or suggestion that PEPA (pentafluoropropionic acid: CF_3CF_2-COOH) without any protic solvent such as methanol (CH_3OH) would be used as a reactant for the degradation of the oxazolone-ring.

At the least, Tsugita fails to provide any teaching or suggestion that the degradation of the oxazolone-ring would be made by using the vapor of PFPMe in the absence of any vapor of protic solvent such as methanol.

The C-terminal amino acid isolated in the form of free amino acid was modified with fluorescien isothiocyanate, and then analyzed by HPLC. The reaction scheme for the modification with fluorescein isothiocyanate may be shown as follows.

<u>Modification with fluorescien isothiocyanate (Flrsc-N=C=S):</u>



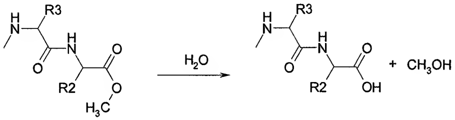
On the other hand, the esterified peptide collected from the reaction solution was subjected to the final reaction.

(iii) The final reaction sub-step for conversion of the esterified peptide (peptidyl methyl ester) into the peptide with a free carboxyl group at its C-terminus:

10 % DMAE aqueous solution was used at 60 °C for 20 min in the hydrolysis reaction of the ester bond to convert the esterified peptide (peptidyl methyl ester) into the peptide with a free carboxyl group.

The reaction of hydrolysis of ester may be carried out by the following reaction scheme:

Hydrolysis of ester:



The peptide with a free carboxyl group was collected from the aqueous solution and was then dried up to be used as a dried peptide sample for the next degradation step.

Accordingly, the peptidyl reaction product (peptide with a free carboxyl group), which is obtained in each of the C-terminal degradation steps, is by no means analyzed by mass spectroscopy.

Indeed, the C-terminal sequence of the denatured protein was made based on the HPLC analysis of the C-terminal amino acid modified with fluorescein isothiocyanate.

Therefore, the dried peptide sample, which has the C-terminal amino acid of –NH-CH(R2)-COOH to be analyzed in the next step of the C-terminal degradation, should be free from such contamination of the denatured protein that retains un-reacted C-terminal amino acid of –NH-CH(R1)-COOH.

At the least, Tsugita fails to teach or suggest any process for preparation of such a mixture comprising the denatured protein and the series of the peptidyl reaction products (peptide with a free carboxyl group), each of which products has a step-wisely decreased

amino acid sequence. Tsugita by no means uses FAB-MS or MALDI-TOF-MS for the process as disclosed in "2.13 C-terminal sequencing".

At the least, Tsugita fails to provide any teaching or suggestion that acetic anhydride would be successfully used for degradation of the oxazolone ring in the presence of PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3).

Further, Tsugita also teaches another process for chemical specific cleavage and multi-point C-terminal sequencing (i.e. "3.1 Chemical specific cleavage and multiple C-terminal sequencing"), which process is carried out on a dried protein as well as on a polyacrylamide gel.

The process for the multi-point C-terminal sequencing for the protein sample on the polyacrylamide gel comprises the following steps (a) - (c):

(a) Step of electro-blotting the protein on the polyacrylamide gel to the Immobilon-CD membrane:

At first, the proteins were subjected to one-dimensional or two dimensional electrophoresis on the polyacrylamide gel. The resultant protein spots on the polyacrylamide gel were electroblotted to the Immobilon-CD membrane and negatively strained. The protein spot identified on the Immobilon-CD membrane was excised and cut into a 1 mm square.

(b) Step of chemical specific cleavage of protein on the blotted membrane:

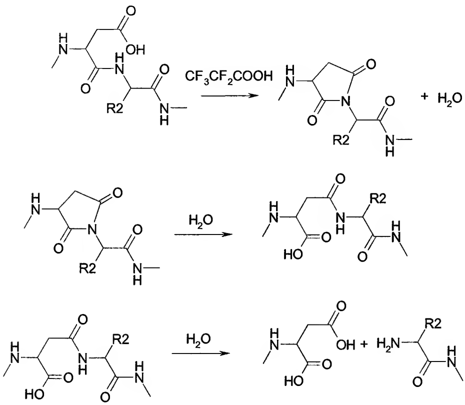
The cut-off square piece of the blotted membrane was put in the small tube and subjected to the specified cleavage reactions. In the specified cleavage reactions, the protein sample was cleaved at the carboxyl side of the aspartyl peptide bond (Asp-C) as disclosed in the Section "3.2 Aspartic acid carboxyl side (Asp-C) cleavage", or at the amino side of the serine or threonine

(Ser/Thr-N) peptide bonds as disclosed in the Section "3.3 Amino side of serine/threonine (Ser-N) cleavage", under the specified cleavage conditions, respectively.

Tsugita employs a specified cleavage condition for the Asp-C cleavage reaction that a vapor phase reaction was created with a vapor generated from a 0.2 % PFPA aqueous solution containing 1 % w/v DTT at 90 °C for 4-16 h. The vapor generated from the 0.2 % PFPA aqueous solution containing 1 % w/v DTT at 90 °C contains a vapor of PFPA, a vapor of H_2O and a vapor of DTT. (See "2.7 Asp-C cleavage of protein")

The group of peptidyl reaction products (peptide fragments) produced in the reaction of cleavage at the carboxyl side of the aspartyl peptide bond (Asp-C) will consist of the N-terminal peptide fragment having a newly exposed C-terminal aspartic acid, inner peptide fragments having a newly exposed C-terminal aspartic acid and the C-terminal peptide fragment.

The Asp-C cleavage reaction may be made through the following reaction scheme.



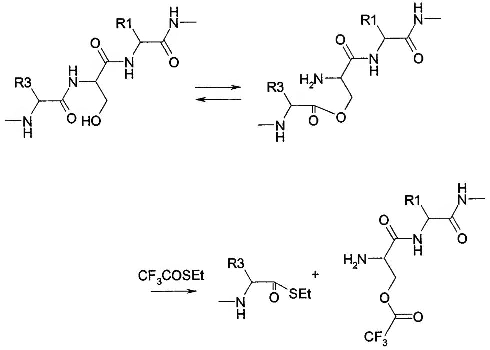
The hydrolysis of the imido structure and the hydrolysis of the amido bond are both catalyzed by the vapor of PFPA.

Further, Tsugita teaches and suggests the additional reaction where the Asp-C cleavage activates the newly exposed C-terminal aspartic acid, accompanied by the liberation the C-terminal aspartyl residue, which reaction is made with a vapor generated from a 0.2 % PFPA aqueous solution containing 1 % w/v DTT at 90 °C.

Tsugita also teaches a specified cleavage condition for the Ser/Thr-N cleavage reaction where a vapor phase reaction is made with a vapor of TFASEt (S-Ethyl trifluorothioacetate; $CF_3CO\text{-}S\text{-}CH_2CH_3$) at 30 °C for 24 h or at 50 °C for 6-24 h. (See "2.8 Ser/Thr-N cleavage of protein" of Tsugita)

The group of peptidyl reaction products (peptide fragments) produced in the reaction of cleavage at the amino side of the serine or threonine (Ser/Thr-N) peptide bonds will consist of the N-terminal peptide fragment, inner peptide fragments having a newly exposed N-terminal Ser/Thr residue and the C-terminal peptide fragment having a newly exposed N-terminal Ser/Thr residue.

The Ser/Thr-N cleavage reaction using the vapor of TFASEt may be made through the following reaction scheme.



Tsugita emphasizes that the TFASEt vapor must be generated from the reagent itself, because the vapor generated from its organic or aqueous solutions results in trifluoroacetylation and/or a decrease in cleavage extent and loss of reaction specificity.

(c) Step of extraction of the peptidyl reaction products from the cut-off square piece of the membrane.

After the specified cleavage reaction, the peptidyl reaction products (peptide fragments) were extracted with 30% and 60% acetonitrile aqueous solutions. The extract was dried and analyzed by FAB-MS or MALDI-TOF-MS.

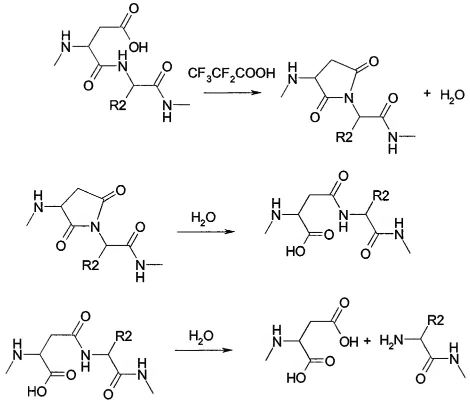
However, Tsugita fails to teach any process in which chemical specific cleavage was carried out on the protein being maintained in a state that it is bound on the polyacrylamide gel in place of the protein on the blotted membrane.

In view of these facts, Tsugita fails to provide any teaching or suggestion as to a process for C-terminal stepwise degradation or for chemical specific cleavage, which is carried out on the peptide (denatured protein) being maintained in a state that it is bound on the gel carrier, in particular on the polyacrylamide gel as recited in Claims 1, 18 and all claims depending therefrom of the present application.

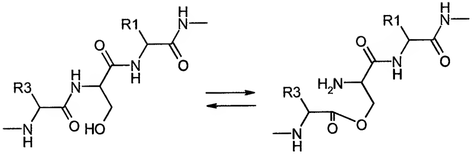
Tsugita fails to provide any teaching or suggestion as to a process for C-terminal stepwise degradation, which is can be used for preparation of a mixture comprising the denatured protein and the series of the peptidyl reaction products (peptide with a free carboxyl group), each of which products has a step-wisely decreased amino acid sequence.

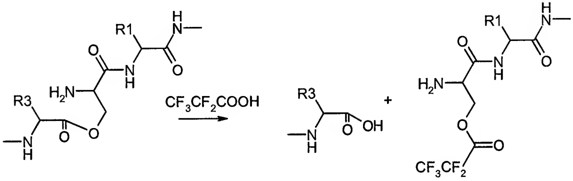
In addition, Tsugita teaches a specified cleavage condition for the Asp-C cleavage reaction and Ser/Thr-N cleavage reaction along with the simultaneous successive C-terminal truncation reaction. These vapor phase reactions were made with a vapor of 90% PFPA (CF_3 CF_2COOH) aqueous solution containing 1% DTT at 90 ºC for 1-16h, as disclosed in the Section "3.4 C-terminal sequencing at multiple sites". The vapor generated from the 90 % PFPA aqueous solution containing 1 % w/v DTT at 90 ºC may contain a vapor of PFPA, a vapor of H_2O and a vapor of DTT. (See "3.4 C-terminal sequencing at multiple sites" of Tsugita)

The Asp-C cleavage reaction may be made by using the vapor generated from the 90 % PFPA aqueous solution through the following reaction scheme.

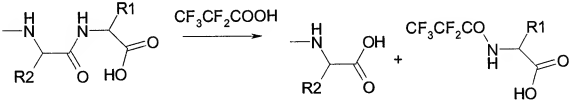


The Ser/Thr-N cleavage reaction may be made by using the vapor generated from the 90 % PFPA aqueous solution at 90 °C through the following reaction scheme.



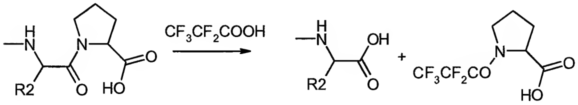


The simultaneous successive C-terminal truncation reaction may be made by using the vapor generated from the 90 % PFPA aqueous solution at 90 °C through the following reaction scheme.



As can be seen, peptides of TQAGRDSFRESLSAL or of SFRESLSAL are formed from the peptides of TQAGRDSFRESLSALP or of SFRESLSALP by removing Pro in the simultaneous successive C-terminal truncation reaction (See Table 3 of Tsugita). **Thus, in Tsugita, the C-terminal Pro residue can never be converted into the oxazolone-ring form.**

Therefore, the C-terminal Pro residue is removed by using the vapor generated from the 90 % PFPA aqueous solution at 90 °C through the following reaction scheme.



These experimental results show that where high vapor pressure of PFPA generated from the 90 % PFPA aqueous solution at 90 °C is used, the liberation of the C-terminal amino acid residue may be caused by the direct cleavage reaction due to PFPA rather than a reaction path through formation of an oxazolone ring.

In contrast, if low vapor pressure of PFPA generated from the 0.2 % PFPA aqueous solution at 90 °C is used, any liberation of the C-terminal amino acid residue other than the newly exposed C-terminal aspartic acid is not observed. (See Table 1 of Tsugita) This experimental evidence also supports the aforementioned direct cleavage reaction scheme due to high vapor pressure of PFPA at 90 °C.

On the other hand, Tsugita teaches a procedure for the delocking of N-acetyl Ser/Thr, in which delocking of N-acetyl Ser/Thr is carried out by exposing protein on a PVDF membrane to a 75% PFPA aqueous vapor at 50 °C for 1 h. In the deblocking reaction for N-acetyl Ser/Thr, vapor of PFPA and vapor of water generated from the 75% PFPA aqueous solution at 50 °C are used to selectively remove acetyl groups from the N-terminal amino group of Ser/Thr. Thus, the relatively low vapor pressure of PFPA at 50 °C for 1 h by no means causes any removal of C-terminal amino acids from the peptide. The reaction scheme for deblocking of N-acetyl Ser/Thr may use the N, O-acyl

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rearrangement reaction catalyzed by vapor of PFPA, and hydrolysis reaction of the ester bond (O-acetylated Ser/Thr: CH_3CO-O-CH_2-) catalyzed by vapor of PFPA. This deblocking reaction for N-acetyl Ser/Thr also provides evidence suggesting that such high vapor pressure of PFPA generated from the 90 % PFPA aqueous solution at 90 °C is required to initiate the aforementioned direct cleavage reaction scheme for C-terminal sequencing at multiple sites.

Further, Tsugita fails to provide any teaching or suggestion as to whether or not PFPA without any protic solvent, which is suitably used in the vapor phase reaction for the Asp-C cleavage reaction, would be employed as a reactant for the liquid phase reaction for degradation of the oxazolone-ring, in place of PFPMe with a protic solvent such as methanol (CH_3OH). At least, the function of PFPMe used in the liquid phase reaction for degradation of the oxazolone-ring is quite different from the catalytic function of PFPA used in the vapor phase reaction for the Asp-C cleavage reaction. Therefore, there is no good reason to believe that PFPA would have a similar function to that of PFPMe used in the liquid phase reaction for degradation of the oxazolone-ring.

As explained above, the reaction scheme of the process used for C-terminal sequencing at multiple sites is concluded to be quite different from the reaction scheme of the process as recited in Claims 1, 18 and all claims depending therefrom of the present application.

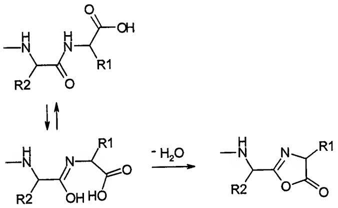
Accordingly, Tsugita fails to teach or suggest any process for preparing a mixture containing a series of peptidyl reaction products by chemically releasing the C-terminal amino acids successively, in which the oxazolone-ring is formed from the C-terminal amino acid, and any chemically cleavage of the peptide is successfully prevented in the chemical

reaction step as recited in Claims 1, 18 and all claims depending therefrom of the present application.

In contrast, the process for releasing the C-terminal amino acids successively from the peptide of the present invention is carried out through the following reaction schemes:

(I) reaction for formation of 5-oxazolone ring:

The reaction for formation of 5-oxazolone ring is expressed on the whole by the following reaction scheme (I):

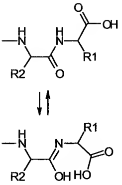


(I)

The reaction of scheme (I) consists of the following two stages (Ia) and (Ib).

(Ia) keto-enol tautomerism:

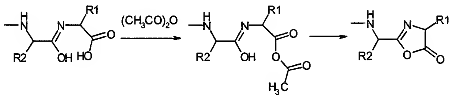
The perfluoroalkanoic acid contained in the mixed solution of the alkanoic acid anhydride and the perfluoroalkanoic acid dissolved in the dipolar aprotic solvent is allowed to act as a proton donor on the dried peptide at the stage of keto-enol tautomerism, as shown in the following reaction scheme (Ia):



(Ia)

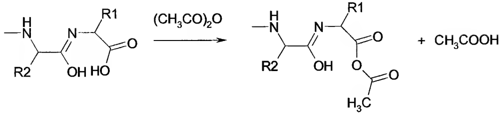
(Ib) formation of the activated C-terminal carboxyl group and formation of the intra-molecular ester bond (formation of the 5-oxazolone ring):

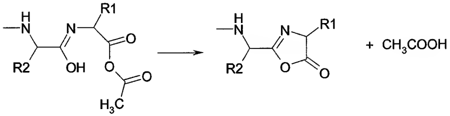
The alkanoic acid anhydride is used as a reagent for formation of the activated C-terminal carboxyl group. The activated C-terminal carboxyl group is reacted with the hydroxyl group to form the 5-oxazolone ring.



(Ib)

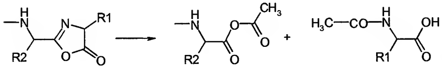
The following is a detailed reaction scheme of the stage (Ib):



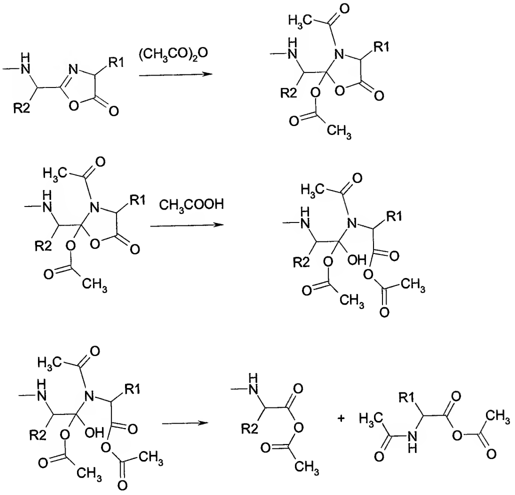


(II') separation of the C-terminal amino acid and formation of the reaction intermediate for the next stage:

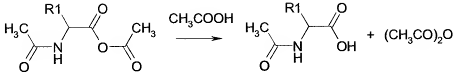
The alkanoic acid anhydride is used as a reagent for the addition reaction on the double bond of >C=N- type of the 5-oxazolone ring. The degradation of the 5-oxazolone ring is made via such a reaction as shown by the following reaction scheme (II'):


(II')

The following may be a detailed reaction scheme of the stage (II').



The alkanoic acid, which is a by-product from the alkanoic acid anhydride formed at the stage (Ia) is used as a reagent at the second reaction for opening of the ester bond therein. In addition, the alkanoic acid also reacts on the derivative of the C-terminal amino acid having acid anhydride form, and thereby the C-terminal acid anhydride form thereof is converted into the C-terminal carboxyl group.



The peptidyl reaction product having the activated C-terminal carboxyl group of the third reaction is ready for the formation of the 5-oxazolone ring at the next stage.

The considerable variation of the reaction speeds of those stages is successfully used to prepare a mixture comprising the original peptide and the series of peptidyl reaction products produced therefrom.

At the least, the reaction schemes used in the process for releasing the C-terminal amino acids successively from the peptide of the present invention are quite different from those used in the process for C-terminal stepwise degradation or in the process for C-terminal sequencing at multiple sites as taught and suggested in Tsugita.

In addition, Tsugita provides experimental evidence suggesting that the MS spectra of the resulted peptides obtained from the process for C-terminal sequencing at multiple sites may lack some peaks due to the series of peptides, for example, the peaks due to fragments of 151-161 and 151-160 from bovine carbonic anhydrase II are not observed for the samples prepared by reaction for 4h, and thus, at least, in the series of the fragments of 151-163 to 151-158, the fragment of 151-161 is not identified from multi C-terminal sequence data. (See Table 3 of Tsugita)

Tsugita suggests that the process for C-terminal sequencing at multiple sites may sometimes fail to analyze the C-terminal sequence of the peptide fragments based on the decrease in the molecular weight of the series of fragments.

Tsugita also does not teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by

using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, Tsugita does not teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Turning to Covey, Covey teaches a procedure of enzymatic digestion of a long peptide by trypsin to cleave the long peptide into tryptic fragments.

Covey also teaches a double charge rule that the tryptic fragment having Arg or Lys at the C-terminus thereof will be doubly positively charged in the form of $(M+2H)^{2+}$ by using Ion spray process for Ion Evaporation Mass Spectrometry, but that there are three exceptions to the double charge rule as follows:

First exception: a tryptic fragment having another amino acid other than Arg or Lys at the C-terminus thereof will only be singly charged as $(M+H)^{+}$ by using Ion spray process for Ion Evaporation Mass Spectrometry.

Second exception: a tryptic fragment having an amino terminus which is carboxylated or blocked (e.g. N-acylation at the N-terminus) will only be singly charged as $(M+H)^{+}$ by using Ion spray process for Ion Evaporation Mass Spectrometry.

Third exception: a tryptic fragment having Arg or Lys at the C-terminus thereof and containing an internal His will be triply charged as $(M+3H)^{3+}$ in a small percentage, but will be doubly charged as $(M+2H)^{2+}$ in the largest percentage.

At the least, Covey fails to provide any teaching or suggestion as to intensity of a singly positive charged ion of $(M+H)^+$ from the tryptic fragment having Arg or Lys at the C-terminus thereof to be measured by Ion Evaporation Mass Spectrometry. Further, Covey fails to provide any teaching or suggestion as to whether or not such a double charge rule will be also observed for MALDI-TOF-MS or FAB-MS.

Covey also fails to provide any teaching or suggestion as to intensity of a singly positive charged ion of $(M+H)^+$ from the tryptic fragment having Arg or Lys at the C-terminus thereof to be measured by MALDI-TOF-MS. Covey fails to provide any teaching or suggestion as to intensity of a singly negative charged ion of $(M-H)^-$ from the tryptic fragment having other amino acids than Arg or Lys at the C-terminus thereof to be measured by MALDI-TOF-MS.

Covey further fails to provide the teaching or suggestion that a singly positive charged ion of $(M+H)^+$ from the tryptic fragment having Arg or Lys at the C-terminus thereof may show stronger intensity in the spectrum of the cationic species of $(M+H)^+$ measured by MALDI-TOF-MS.

More detailed explanation as to the technique of Covey is provided as follows:

At first, Covey uses a typical process, which comprises the steps of:

1) digesting the target protein with trypsin to produce a mixture of the tryptic fragments containing only one Arg or Lys at its C-terminus and the C-terminal fragment containing no Arg of Lys;

2) separating each of the fragments from the mixture of the fragments produced in step 1) by using chromatographic separation procedure such as HPLC;

3) separately mass-analyzing each of the fragments, which are separated from each other in step 2), by using Ion Evaporation Mass Spectrometry,

wherein each of the fragments is mass-analyzed at the different scan, in which such a liquid sample of each of the fragments solved in an acidic aqueous solvent is ionized by ion evaporation.

In the acidic aqueous solvent such as 0.5% formic acid or 0.1% trifluoroacetic acid aqueous solution, the tryptic fragments containing only one Arg or Lys at its C-terminus is solved in such a doubly ionized cationic form, in which the carboxyl groups are not ionized, the N-terminal amino group is ionized in the form of $-NH_3^+$, and the amino group of Lys at the C-terminus is ionized in the form of $-NH_3^+$ or the guanidino group ($-NH-C(NH_2)=NH$) of Lys at the C-terminus is ionized in the form of $-NH-C^+(NH_2)-NH_2$.

In the acidic aqueous solvent such as 0.5% formic acid or 0.1% trifluoroacetic acid aqueous solution, the C-terminal fragment containing no Arg of Lys is solved in such a simply ionized cationic form, in which the carboxyl groups are not ionized and the N-terminal amino group is ionized in the form of $-NH_3^+$. Therefore, in the ionization process using ion evaporation, the doubly ionized cation of the tryptic fragments containing only one Arg or Lys at its C-terminus that is solved in the acidic aqueous solvent will be converted into the doubly ionized cationic species of $(M+2H)^{2+}$ in gas phase at high efficiency of 90-100%. The doubly ionized cation of the tryptic fragments containing only one Arg or Lys at its C-terminus that is solved in the acidic aqueous solvent may be converted into the singly ionized cationic species of $(M+H)^+$ in gas phase at a low efficiency. However, the doubly ionized cation of the tryptic

fragments containing only one Arg or Lys at its C-terminus that is solved in the acidic aqueous solvent will not be converted into such a singly ionized anionic species of $(M-H)^-$ in gas phase by ion evaporation.

On the other hand, the singly ionized cation of the C-terminal fragments containing no Arg or Lys that is solved in the acidic aqueous solvent will be converted into the singly ionized cationic species of $(M+H)^+$ in gas phase at a high efficiency. However, the singly ionized cation of the C-terminal fragments containing no Arg or Lys that is solved in the acidic aqueous solvent may not be converted into the singly ionized anionic species of $(M-H)^-$ in gas phase by ion evaporation.

When each of the tryptic fragments containing only one Arg or Lys at its C-terminus and the C-terminal fragment containing no Arg of Lys solved in the acidic aqueous solvent is applied to the Ion Evaporation Mass Spectrometry, only the cationic species will be observed in the positive mode spectrum, whereas any anionic species will be rarely observed in the negative mode spectrum. Further, each of the tryptic fragments containing only one Arg or Lys at its C-terminus is separated from the mixture of the fragments by using chromatographic separation procedure such as HPLC, so that only one doubly ionized cationic species of $(M+2H)^{2+}$ in gas phase that is converted from the doubly ionized cation of each of the tryptic fragments containing only one Arg or Lys at its C-terminus solved in the acidic aqueous solvent is observed at the position of $m/z=(M+2)/2$ in each scan by the Ion Evaporation Mass Spectrometry, as shown in FIG. 4A, 4B and 4C of Covey.

If the tryptic fragment containing only one Arg or Lys at its C-terminus further contains His residue, the tryptic fragments containing only one Arg or Lys at its C-terminus and His residue may be solved in such a triply ionized cationic form at a high percentage as well as in the

doubly ionized cationic form at a high percentage. In such a case, additional triply ionized cationic species of $(M+3H)^{3+}$ in gas phase that is converted from the triply ionized cation of the tryptic fragments containing only one Arg or Lys at its C-terminus and His residue solved in the acidic aqueous solvent is observed at the position of m/z=(M+3)/3 by the Ion Evaporation Mass Spectrometry, as shown in FIG. 5 of Covey.

The process disclosed in Covey further comprises a second stage of mass analysis in the known MS-CID-MS mode. In the MS-CID-MS mode, the doubly ionized cationic species of $(M+2H)^{2+}$ in gas phase, that is converted from the doubly ionized cation of each of the tryptic fragments containing only one Arg or Lys at its C-terminus solved in the acidic aqueous solvent, is subjected to collision induced dissociation in order to produce daughter ions from the doubly ionized cationic species of $(M+2H)^{2+}$. In the step of collision induced dissociation, the doubly ionized cationic species of $(M+2H)^{2+}$ is dissociated into a couple of daughter ions, i.e. a singly ionized cationic species of $(M_N+H)^+$ that is N-terminal daughter ion containing the N-terminal amino group ionized in the form of $-NH_3^+$, and a singly ionized cationic species of $(M_C+H)^+$ that is C-terminal daughter ion containing only one Arg or Lys with $-NH-C^+(NH_2)-NH_2$.or $-NH_3^+$ at its C-terminus, wherein $M_N + M_C = M$.

Thus, if the spectrum in the MS-CID-MS mode is measured for each of tryptic fragments containing only one Arg or Lys at its C-terminus, plurality of couples of daughter ions, such as Y11 and B1, …, Y1 and B11 as illustrated in FIG.7, are observed around the position of m/z=(M+2)/2 for the parent ion that is the doubly ionized cationic species of $(M+2H)^{2+}$. The fact that some of the daughter ions are observed at positions of m/z larger than (M+2)/2 provides good evidence proving that the parent ion observed at the position of m/z=(M+2)/2 is indeed the doubly ionized cationic species of $(M+2H)^{2+}$, whereas a couple of daughter ions observed at

m/z= (M_N + 1) and m/z= (M_C + 1) are a singly ionized cationic species of $(M_N+H)^+$ and a singly ionized cationic species of $(M_N+H)^+$, respectively. Such feature of the spectrum observed in the MS-CID-MS mode for each of tryptic fragments containing only one Arg or Lys at its C-terminus is illustrated in FIG. 8 of Covey.

Accordingly, the process disclosed in Covey employs the spectrum observed in the MS-CID-MS mode in order to prove that the parent ion, which is observed at the position of m/z=(M+2)/2 in the spectrum of each scan by the Ion Evaporation Mass Spectrometry, is indeed the doubly ionized cationic species of $(M+2H)^{2+}$. Further, each of the couples of daughter ions, such as Y11 and B1, ..., Y1 and B11 as illustrated in FIG.7 of Covey, is assigned in the spectrum measured in the MS-CID-MS mode by using a criteria where a couple of daughter ions are observed at m/z= (M_N + 1) and m/z= (M_C + 1), wherein $M_N + M_C = M$, whereas the parent ion that is the doubly ionized cationic species of $(M+2H)^{2+}$ is observed at the center position of m/z=(M+2)/2, as illustrated in FIG. 8 of Covey.

Covey by no means uses any spectrum of anionic species in assignment of each of the fragments that is separated from the mixture of the fragments produced in the step 1), as no anionic species is observed for each of the fragments solved in the acidic aqueous solvent by the Ion Evaporation Mass Spectrometry.

Covey also does not teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, Covey does not teach or suggest a hydrolysis reaction being carried out by using water

molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Xu teaches a procedure of structural characterization of muropeptides derived from peptidoglycan by means of MALD-MS based method. Xu reported the MALDI-TOF-MS spectra measured for the muropeptides derived from peptidoglycan by using lysostaphin digestion such as the positive-ion linear MALDI mass spectra shown in Figure 2 of Xu, in which $[M+Na]^+$ ion peaks are the dominant species, but $[M+H]^+$ ion peaks are not measured at any detectable level.

In particular, in MALDI-PSD analysis of an un-substituted monomer, Xu. clearly stated "In positive-ion mode analysis, the $[M+Na]^+$ ion at m/z=991 was selected as the precursor for an unsubstituted muropeptide monomer because of its high abundance relative to the protonated molecules $[M+H]^+$." <u>See</u> page 10 right hand column of Xu.

Xu fails to teach or suggest any use of the protonated molecules $[M+H]^+$ for the positive-ion mode analysis. Accordingly, Xu fails to provide any teaching or suggestion that the protonated molecules $[M+H]^+$ will be used for structural characterization of peptides in combination with the deprotonated molecules $[M-H]^-$.

A more detailed explanation of the technique of Xu is given as follows:

At first, there is monomeric and dimeric muropeptides which have been previously identified by FAB-MS and FAB-MS/MS. In addition, the amino acid compositions of each of

muropeptides were determined in advance to the structural characterization by mass-spectrometry. Thus, the structures of pentaglycine-substituted monomers and the muropeptide oligomers can be reasonably predicted on the basis of the reported structures of the monomeric and dimeric muropeptides. The structure analysis of muropeptides was made using mass-spectrometry by referring the well predicted structures of muropeptides, as presented in FIG. 1 of Xu. The muropeptide samples were digested with lysostaphin in 12.5 mM sodium phosphate buffer (pH 5.5) for 16 h and analyzed directly without further purification. Thus, the analyte solution used for the MALDI analysis contains mixture of the fragments having N-terminal amino group of Gly residue included in a polyglycine chain substituted on the Lys residue and the C-terminal carboxyl group of Ala (or Gly) at the C-terminus of the main peptide chain in the 12.5 mM sodium phosphate buffer (pH 5.5).

The samples for mass spectrometric analysis were typically prepared by depositing about 1 μl of CMBT solution and about 1 μl of the analyte solution on a sample plate well and mixing. The solvents were removed by air drying. Thus, in the dried-up samples, most of the fragments may be present in such a salt form in which the C-terminal carboxyl group of Ala (or Gly) at the C-terminus of the main peptide chain is converted into $-COO^{-}Na^{+}$, whereas few of the fragments may be present in such a form in which the C-terminal carboxyl group of Ala (or Gly) at the C-terminus of the main peptide chain is converted into –COOH. The singly ionized cationic species of $[M+Na]^{+}$ is predicted to be a cation in which the N-terminal amino group of Gly residue included in the polyglycine chain is ionized in the form of $-NH_3^{+}$, and the C-terminal carboxyl group of Ala (or Gly) at the C-terminus of a main peptide chain is converted into $-COO^{-}Na^{+}$.

On the other hand, a protonated molecule of $[M+H]^+$ is predicted to be a cation in which the N-terminal amino group of Gly residue included in the polyglycine chain is ionized in the form of $-NH_3^+$, and the C-terminal carboxyl group of Ala (or Gly) at the C-terminus of the main peptide chain is present in the form of $-COOH$. The singly ionized anionic species of $[M-H]^-$ is predicted to be a cation in which the N-terminal amino group of a Gly residue included in the polyglycine chain is present in the form of $-NH_2$, and the C-terminal carboxyl group of Ala (or Gly) at the C-terminus of the main peptide chain is ionized in the form of $-COO^-$.

Accordingly, in the Negative-ion MALDI mass spectrum of the Lysostaphin digest of multipeptides, only the single peak generated from each of the fragments is observed at the position of m/z=(M-1). On the other hand, in the Positive-ion MALDI mass spectrum of the Lysostaphin digest of multipeptides, the series of peaks of the singly ionized cationic species of $[M+Na]^+$ and the series of peaks of the singly ionized cationic species of $[M+H]^+$ will be both observed at the position of m/z=(M+23) and at the position of m/z=(M+1) in parallel. Therefore, the Negative-ion MALDI mass spectrum of the Lysostaphin digest of multipeptides is suitably used to identify the mass of each of the fragments contained in the analyte solution, by referring the well predicted structures of muropeptides, as illustrated in FIG. 6 and TABLE 1 and in FIG. 7 and TABLE 2 of Xu.

In contrast, the collected muropeptides that are separated by HPLC were desalted with acetonitrile, and thus, the desalted muropeptides samples are present in a form in which the N-terminal amino group of Gly residue included in polyglycine chain is present in the form of $-NH_2$, and the C-terminal carboxyl group of Ala (or Gly) at the C-terminus of the main peptide chain is present in the form of $-COOH$.

Thus, in the dried-up samples of the desalted muropeptides used for the mass spectrometric analysis, the desalted muropeptides are also present in such a form, in which the N-terminal amino group of Gly residue included in the polyglycine chain is present in the form of $-NH_2$, and the C-terminal carboxyl group of Ala (or Gly) at the C-terminus of main peptide chain is present in the form of $-COOH$.

Therefore, in the Positive-ion MALDI mass spectrum of the desalted muropeptides, only a single peak of the singly ionized cationic species of $[M+H]^+$ will be observed at the position of $m/z=(M+1)$, but any peak of such a singly ionized cationic species of $[M+Na]^+$ may not be observed. Of course, in the Negative-ion MALDI mass spectrum of the desalted muropeptides, only a single peak of the singly ionized anionic species of $[M-H]^-$ will be observed at the position of $m/z=(M-1)$. Accordingly, the molecular weight of the desalted muropeptide dimer I was determined as 2417 by both positive- and negative-mode MALDI-MS.

However, Xu fails to teach or suggest that the spectrum observed in the positive mode would be suitably used in combination with the spectrum observed in the negative mode to identify the mass of each of the fragments contained in the analyte solution, rather than single use of the spectrum observed in the negative mode. Further, Xu teaches and suggests MALDI-PSD analysis, in which peaks of the daughter ions such as $[b_{n-1} + Na + OH]^+$, $[b_n + Na - H]^+$ and $[a_n + Na - H]^+$ are measured in the positive-ion MALDI-PSD mass spectrum, but peaks of the daughter ions as $[b_n + H]^+$ or $[a_n + H]^+$ are not measured in the positive-ion MALDI-PSD mass spectrum. In contrast, peaks of the daughter ions as $[M - H]^-$ are measured in negative-ion MALDI-PSD mass spectrum.

Therefore, Xu fails to provide any teaching or suggestion as to a MALDI-PSD analysis protocol in which the combination of such peaks of the positive charged daughter

ions as $[M+H]^+$ and such peaks of the negative charged daughter ions as $[M-H]^-$ are used to identify the daughter fragments generated from the post-source decay process of the parent peptide.

Further, Xu teaches correlating peaks as the peaks of the daughter ions $[b_n + Na + OH]^+$ and $[b_n + Na - H]^+$ measured in positive-ion MALDI-PSD mass spectrum of FIG. 3 and FIG. 4 of Xu, and also teaches that the peak of the daughter ion as $[b_n + Na + OH]^+ = 920$ is not associated with any peak of the daughter ion as $[b_n + Na - H]^+$ as shown in FIG. 3 of Xu.

In view of this fact, Xu fails to provide any teaching or suggestion that the peaks of the daughter ions as $[b_n + Na + OH]^+$ may be often associated with the peaks the daughter ions as $[b_n + Na - H]^+$.

Xu also does not teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, Xu does not teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

The combination of Tsugita, Covey and Xu does not render the claimed invention obvious for at least the following reasons. None of the references teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, none of the references teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Therefore, the combination of Tsugita, Covey and Xu does not teach or suggest these recited elements. Since the combination of Tsugita, Covey and Xu does not teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids or a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide

having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, the combination of references do not render the claimed invention obvious. Thus, it is respectfully requested that the rejection of Claims 1, 2, 4-6 and 18 under 35 U.S.C. §103(a) be withdrawn.

Claim 3 stands rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Tsugita in view of Covey and Xu, in further view of Harris. The deficiencies of Tsugita, Covey and Xu are discussed above. Harris does not cure these deficiencies. This rejection should be withdrawn based on the comments and remarks herein.

Harris teaches and suggests a method of using the $[M+H]^+$ ions from trypsin autolysis fragments as mass calibrants in a positive-ion mode MALDI-TOF based analysis.

However, Harris fails to provide any experimental evidence suggesting that the $[M-H]^-$ ions from the trypsin autolysis fragments will be successfully used as mass calibrants in the negative-ion mode MALDI-TOF based analysis. At the least, Harris fails to provide any experimental evidence suggesting that the $[M-H]^-$ ions from the trypsin autolysis fragments will be successfully measured in the negative-ion mode MALDI-TOF based analysis.

Harris also does not teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, Harris does not teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of

reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

The combination of Tsugita, Covey, Xu and Harris does not render the claimed invention obvious for at least the following reasons. None of the references teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, none of the references teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

None of the references, Tsugita, Covey, Xu or Harris teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Further, none of the references teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Therefore, the combination of Tsugita, Covey, Xu and Harris does not teach or suggest these recited elements. Since the combination of Tsugita, Covey, Xu and Harris does not teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids or a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, the combination of references do not render the claimed invention obvious. Thus, it is respectfully requested that the rejection of Claim 3 under 35 U.S.C. §103(a) be withdrawn.

Claims 7-17 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Tsugita in view of Covey and Xu and in further view of Vogt. The deficiencies of Tsugita,

Covey and Xu are discussed above and herein below. Vogt does not cure these deficiencies. This rejection should be withdrawn based on the comments and remarks herein.

Regarding the primary reference, Tsugita fails to teach any process in which chemical specific cleavage was carried out on the protein being maintained in a state that it is bound on the polyacrylamide gel in place of the protein on the blotted membrane. Accordingly, Tsugita fails to provide any suggestion as to a process for C-terminal stepwise degradation or for chemical specific cleavage, which is carried out on the peptide (denatured protein) being maintained in a state that it is bound on the gel carrier, in particular on the polyacrylamide gel.

The process as claimed in Claims 7-17 employs a liquid phase reaction in place of a vapor phase reaction. At the least, the reaction schemes used in the process for releasing the C-terminal amino acids successively from the peptide are quite different from those used in the process disclosed in Tsugita.

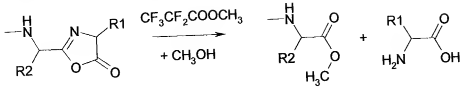
In particular, Tsugita teaches the following reaction for degradation of the oxazolone-ring.

(ii) The second reaction sub-step for degradation of the oxazolone-ring to liberate the C-terminal amino acid and to form the esterified peptide:

The reaction is made with 5% PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3) in methanol (CH_3OH) at 5 °C for 15 min.

The reaction of degradation of the oxazolone may be carried out by the following reaction scheme:

Degradation of the oxazolone:



The reaction mechanism may be alcoholysis in help of catalytic function of PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3. The C-terminal amino acid was liberated to be dissolved in the methanol solution, and thus, the peptidyl reaction product was formed in the shape of esterified peptide. Therefore, PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3) was used as a catalytic agent for inducing the solvolysis reaction with use of methanol (CH_3OH) on the oxazolone-ring.

In view of this fact, Tsugita fails to teach or suggest any reaction for degradation of the oxazolone-ring which would be achieved without methanol (CH_3OH). Furthermore, Tsugita fails to provide any evidence suggesting that PEPA (pentafluoropropionic acid: CF_3CF_2-COOH) without methanol (CH_3OH) would be used as a reactant for the degradation of the oxazolone, in place of PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3) with methanol (CH_3OH). Methanol (CH_3OH) is a well-known protic solvent.

Therefore, Tsugita fails to provide any evidence suggesting that PEPA (pentafluoropropionic acid: CF_3CF_2-COOH) without any protic solvent such as methanol (CH_3OH) would be used as a reactant for the degradation of the oxazolone-ring. Further, Tsugita teaches a procedure of reactions for C-terminal stepwise degradation used for the dried protein sample or protein charged on the minicolumn of C18 silica comprising the aforementioned three reaction sub-steps (i) – (iii).

Tsugita does not teach any step in which acetic anhydride is used in the presence of PFPMe (pentafluoropropionic methyl ester: CF_3CF_2-CO-OCH_3).

Vogt et al. teaches a process for preparation of a high reactive gel-suspension of carboxymethyl cellulose (CMC), in which the polymer (carboxymethyl cellulose) is treated in a dipolar-aprotic solvent, such as N,N-dimethylacetamide and dimethylsulfoxide (DMSO), with p-toluene-sulfonic acid. Vogt et al. also provides an assumed mechanism that the activation (swelling in the dipolar-aprotic solvent) is achieved via an interaction between the carboxylate groups (-CH_2-COONa) of Na-CMC and HO_3S-groups of the p-toluene-sulfonic acid with a rapid exchange of the acidic hydrogen as well as an interaction of the lipophilic toluene unit of the p-toluene-sulfonic acid with the solvent. **Accordingly, Vogt fails to teach any process for preparation of gel-suspension of CMC in the dipolar-aprotic solvent without p-toluene-sulfonic acid.**

Vogt also teaches and suggests that an effective method for activation of CMC is precipitation of an aqueous solution of CMC by N,N-dimethylformamide (DMF) and the removal of the water from the swollen gel by repeated distribution under reduced pressure. This teaching indicates that N,N-dimethylformamide (DMF) can never remove water from the water-swollen gel of CMC. Vogt teaches and suggests that other acids like methane sulfonic acid, trifluoroacetic acid and monochloroacetic acid do not swell CMC to a comparable extent. Furthermore, Vogt teaches and suggests that polysaccharides, directly at the polymer backbone bound carboxy groups like sodium alginate, sodium pectinate, and 6-carboxy cellulose do also not swell in the manner described for CMC.

In view of these teachings Vogt fails to provide any teaching or suggestion as to whether or not the combinational use of the dipolar-aprotic solvent with an acid other than

p-toluene-sulfonic acid is successfully applied for preparation of non-aqueous swelling gel of other polymer than CMC. At the least, Vogt fails to provide any teaching or suggestion as to whether or not the combinational use of the dipolar-aprotic solvent with perfluoroalkanoic acid is successfully applied for preparation of non-aqueous swelling gel of other polymer than CMC.

Further, Vogt fails to provide any teaching or suggestion as to whether or not the use of the dipolar-aprotic solvent without p-toluene-sulfonic acid is successfully applied for preparation of non-aqueous swelling gel of other polymer than CMC. In particular, Vogt fails to provide any teaching or suggestion as to whether or not the use of the dipolar-aprotic solvent without p-toluene-sulfonic acid is successfully applied for preparation of non-aqueous swelling gel of polyacrylamide in quite similar manner to the case of CMC.

Vogt also does not teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, Vogt does not teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

The combination of Tsugita, Covey, Xu and Vogt does not render the claimed invention obvious for at least the following reasons. None of the references teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids as recited in Claims 1, 18 and all claims depending therefrom of the present application. Further, none of the references teach or suggest a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, as recited in Claims 1, 18 and all claims depending therefrom of the present application.

Therefore, the combination of Tsugita, Covey, Xu and Vogt does not teach or suggest these recited elements. Since the combination of Tsugita, Covey, Xu and Vogt does not teach or suggest the formation of a 5-oxazolone structure and the cleavage of the 5-oxazolone ring is carried out in parallel at the same temperature by using the alkanoic acid anhydride in the presence of a catalytic amount of the perfluoroalkanoic acid to successively release the C-terminal amino acids or a hydrolysis reaction being carried out by using water molecules in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound, whereby the mixture containing the series of reaction products having a carboxyl group at their C-terminal as

well as the remains of the original peptide having a carboxyl group at its C-terminus are prepared as a sample to be used for the analysis by means of MALDI-TOF-MS, the combination of references do not render the claimed invention obvious. Thus, it is respectfully requested that the rejection of Claims 7-17 under 35 U.S.C. §103(a) be withdrawn.

For at least the reasons set forth in the foregoing discussion, Applicants believe that the Application is now allowable, and respectfully request that the Examiner reconsider the rejection and allow the Application. Should the Examiner have any questions regarding this Amendment, or regarding the Application generally, the Examiner is invited to telephone the undersigned attorney.

Respectfully submitted,

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